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Cannabinoid receptor subtype 2 (CB2R) in a multitarget approach: perspective of an innovative strategy in cancer and neurodegeneration

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Abstract

The cannabinoid receptor subtype 2 (CB2R) represents an interesting and new therapeutic target for its involvement in the first steps of neurodegeneration as well as in cancer onset and progression. Several studies, focused on different types of tumors, report a promising anticancer activity induced by CB2R agonists due to their ability to reduce inflammation and cell proliferation. Moreover, in neuroinflammation, the stimulation of CB2R, overexpressed in microglial cells, exerts beneficial effects in neurodegenerative disorders. With the aim to overcome current treatment limitations, new drugs can be developed by specifically modulating, together with CB2R, other targets involved in such multifactorial disorders. Building on successful case studies of already developed multitarget strategies involving CB2R, in this perspective we aim at prompting the scientific community to consider new promising target associations involving HDACs (histone deacetylases) and sigma receptors by employing modern approaches based on molecular hybridization, computational polypharmacology and machine learning algorithms.

■ INTRODUCTION

The Cannabinoid receptor subtype 2 (CB2R), together with the subtype 1 (CB1R), belongs to the Endo Cannabinoid System, widely explored because of its physiological functions and more importantly because of its involvement in a wide range of conditions, such as cancer and neurodegeneration.[1,2,3]

Differently from the CB1R subtype, mainly localized at the central nervous system (CNS), where its activation leads to unwanted psychotropic effects,[4] CB2R is physiologically expressed at peripheral level, mainly in the immune system (tonsils, spleen, thymus, lymphocytes, monocytes, macrophages, eosinophils, etc).[5] In pathological conditions, this receptor is overexpressed in the activated microglia at the CNS[6-10] and in several types of cancer.[11-14] Importantly, its activation is responsible for the restoration of normal microglial function as a result of the inhibition of neuroinflammatory signalling pathways.[5]

In the Alzheimer Disease (AD) onset, for instance, CB2R counteracts microglia-mediated neurotoxicity by inhibiting the production of pro-inflammatory mediators and by modulating the migration of macrophages.[10] High levels of CB2R were found in *post-mortem* AD patients brain, in particular in the microglial cells surrounding the β-amyloid plaques.[15] Interestingly, *in vitro* and *in vivo* studies demonstrated the ability of CB2R agonists to reduce both the levels of pre-existing β-amyloid (Aβ) plaques and their formation, leading to a general improvement of memory,[2] as well as promoting intrinsic brain repair mechanisms.[16] Moreover, CB2R activation, together with the decrease in the production, aggregation, and clearance of Aβ plaques, reduces the hyper-phosphorylation of the tau protein, another pathological AD hallmark.[17]

The neuroprotective effects exerted by CB2R activation in AD were also observed in other neurodegenerative disorders such as Parkinson,[2,18] Huntington's disease and Amyotrophic lateral sclerosis.[19] In the oncologic field, a high CB2R expression has been found in several types of cancer (breast, lung, prostate, pancreas and resistant cancers)[20-23] and a correlation between CB2R expression and the histological grade and prognosis has been demonstrated in breast cancer, colon-

rectal carcinoma, glioblastoma and glioma,[24] with different suggested mechanisms.[13] All of these pieces of evidence make CB2R modulators potential therapeutics in different types of pathologic conditions where inflammation is involved such as cancer and neurodegeneration. Furthermore, properly functionalized CB2R ligands can be considered useful molecular probes.[25] Given the multifactorial nature of these diseases, it is today evident that therapies acting on multiple mechanisms are highly desirable. Based on this evidence, multitarget approaches combining CB2R activation with the ability of modulating other targets (e.g. Acetylcholinesterase (AChE) and Butyriylcholinesterase (BChE))[26-31] have been recently proposed to improve AD therapy. As extensively reviewed in the last years, [32–34] the so-called multitarget directed ligands (MTDLs) have several advantages over combination approaches where two or more drugs are administered at the same time.[35] Achieving an optimized activity towards multiple targets, however, is still considered one of the most difficult challenges facing contemporary medicinal chemistry. This is mostly due to the difficulty in preserving drug-like properties while, at the same time, finding a balance between the "wanted polyphamacology" and the "dangerous promiscuity" of the designed drugs. Obviously, this task becomes even more challenging when the targets of interest belong to different protein families. [33]

This issue can be properly approached by integrating different medicinal chemistry strategies, including those based on knowledge-based framework combination (*e.g.* molecular hybridization), computational polypharmacology and machine learning algorithms. [36–38]

Aim of this perspective is to propose a new multitarget approach where CB2R is associated with other important targets involved both in cancer and in neurodegenerative diseases such as Histone deacetylases (HDAC) and sigma receptors.

Noteworthy, these targets were selected being overexpressed[39-47] and linked to inflammation modulation.[48–51]. In details, HDAC inhibitors have been successfully employed as cytotoxic agents in tumours as breast cancer,[41,42] triple negative breast cancer,[43,44] lung,[46,47] pancreatic and prostate cancers,[45] where CB2R involvement has been demonstrated.[20-23] More importantly, CB2R agonists

and HDAC inhibitors share the same effect not only as antiproliferative agents but also as anti-inflammatory modulators, being able to modulate microglia/macrophages polarization from M1 state (pro-inflammatory and detrimental) to the M2 state (anti-inflammatory or pro-resolving).[52-55] The influence on the polarization of microglia/macrophages may be considered as a useful approach to treat some microglia/macrophage-mediated chronic inflammatory diseases as cancer and neurodegeneration.[56]

As for sigma receptors, they are involved in the same types of cancer.[40,39] More specifically, sigma-2 agonists and sigma-1 antagonists display cytotoxic effects similar to those resulting from a CB2R activation. Moreover, the involvement of both sigma receptors on inflammation is widely reported[48-51,57] and interestingly the effect of sigma-1 on microglia polarization has been suggested.[58,59]

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Inspired by successful case studies of already developed multitarget strategies for AD treatment (*i.e.* compounds acting as CB2R agonists and AChE/BChE (Acetylcholinesterase/ Butyrylcholinesterase) inhibitors, in this paper we propose different strategies for designing dual CB2R agonists/HDAC inhibitors (hereinafter referred to as dual CB2R/HDAC) and dual CB2R agonists/sigma modulators (dual CB2R/sigma) based on: i) the evidence of common pharmacophoric elements (molecular hybridization); ii) the presence of valuable structural information concerning the targets of interest (structure-based drug design) and iii) the knowledge, based on preliminary data, of active ligands sharing a common chemical space (ligand-based drug design and machine-learning-assisted *de novo* design).

■ CB2R AGONISTS: THE PROPOSED PHARMACOPHORE

Several classes of compounds acting as CB2R agonists have been developed in the last years, bearing different scaffolds: dibenzopyranic,[60,61] oxoquinoline,[62-65] naphthyridinone,[66–68] quinolinedione,[69] alkyloxy-coumarin,[70] indole,[71–75] indazole,[76] imidazopyridine,[77] imidazopyrazine,[78] benzimidazole,[79,80] purine,[81,82] thiophene,[83,84] triazine,[85,86] pyridinone,[87–89]

biphenyl,[90,91] proline,[92,93] and piperidine.[94] In Figure 1 the most representative and selective known CB2R ligands are reported along with their K_i values on CB1R and CB2R.

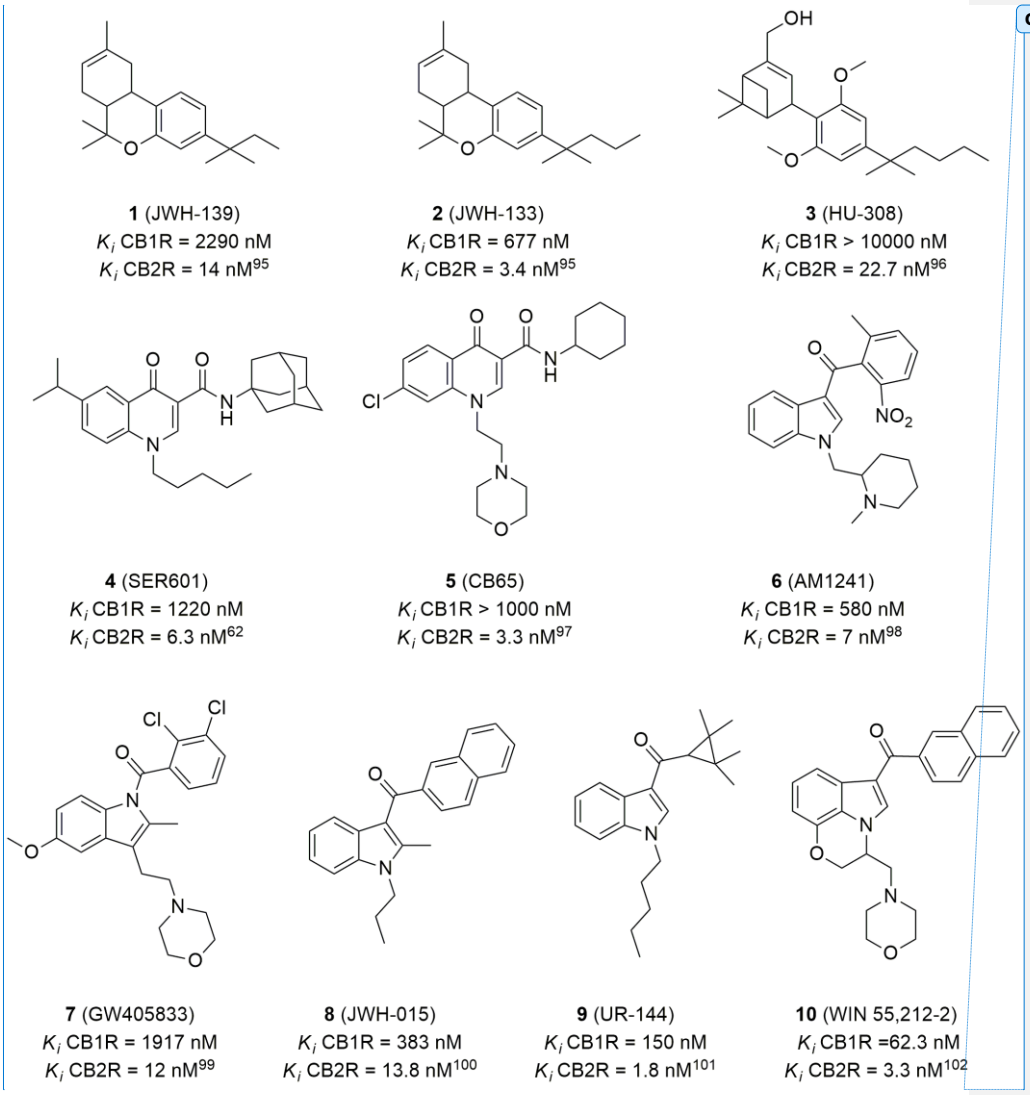

Figure 1. Representative CB2R ligands bearing different scaffolds.[62, 95-102]

Based on the available literature, we recently proposed[20] a CB2R pharmacophore reported in Figure 2.

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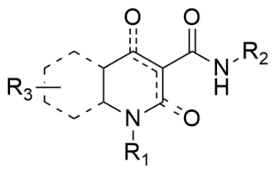

Figure 2. Proposed CB2R pharmacophore.

The first structural requirement is represented by a monocyclic or bicyclic core carrying hetero atoms (N, S, O): a nitrogen in the heterocycle leads to an improved affinity but it is not essential for the activity.[103] The presence of OH or C=O groups in 2- or 4- position of the core increases the affinity *vs* the target *via* H-bonds formation.[2] On the core scaffold, R1 can be: i) a linear alkyl chain (4-6 carbon atoms) often formed by 5-methylenes units (that proved to be optimal for receptor affinity[62-104] due to their ability to maximize the interactions with a hydrophobic region of the receptor); ii) cycloalkyl or aromatic-alkyl groups; iii) heterocycloalkyl ring as alkyl-morpholine or piperidine.[105]

To meet selectivity towards CB1R, in order to avoid the unwanted psychotropic effects due to the interaction with the CB1R subtype, and to increase binding affinity, an aliphatic (cycloalkyl rings) or aromatic (naphthalene, phenyl) carboxamide (R_2) is usually inserted on the core. Noteworthy, the carboxamide portion can be replaced by bioisosters (*e.g*. a carbonyl). R_2 is a bulky and lipophilic group that confers great selectivity and potency. Examples of used R2 groups are represented by the adamantane (strong increase in selectivity), lipophilic cycloalkyls (cyclohexyl, cycloheptyl), branched aliphatic chains (*i*-Pr, *t*-Bu).[106]

In R_3, the presence of aromatic groups that allow the formation of π-π stacking interactions with the aromatic amino acids located in a deep hydrophobic pocket of the receptor may be favourable to the affinity *vs* CB2R. The substitutions on the bicycle may lead to discriminate between agonist and antagonist activity.[68]

■ A SUCCESSFUL CASE STUDY: MOLECULAR HYBRIDIZATION BASED ON CB2R AGONISTS AND ACHE/BCHE INHIBITORS

The molecular hybridization strategy is based on the combination of two molecules endowed with different pharmacological activities in a single chemical entity, able to hit different targets involved in the same multifactorial pathology. The adopted strategies can be based on the connection (through a linker) or the integration (by merging or fusing) of pharmacophoric frameworks. Although very promising to combat the multifactorial nature of complex diseases, the development of molecular hybrids faces the critical issues of selecting the right target combination and the achievement of a balanced activity towards them, while maintaining drug-like-properties for the diverse targets.[107–109]

As suggested by Bolognesi, early ADME studies should be included at early stage as to overcome all the pharmacokinetic issues.[110]

As mentioned above, CB2R activation is widely reported to improve cognitive impairment in animal models of AD, as CB2R agonists may reduce the release of pro-inflammatory molecules, facilitate the clearance of Aβ, promote the phagocytic phenotype of microglia, reduce the Aβ neurotoxicity and reduce the oxidative stress damage produced by reactive oxidative species (ROS) and tau hyperphosphorylation.[15] Cannabinoids are potentially excellent multi-target candidate drugs for their interesting pharmacological profiles. Notable is the double ability to act as CB2R agonist and cholinesterase inhibitors (AChE/BChE) reported for some of them.[26] The AChE and BChE enzymes are identified as critical targets for the effective management of AD.[111] Together with memantine (a N-methyl-D-aspartate (NMDA) receptor antagonist), three cholinesterase inhibitors (AChE/BChE) (Figure 3) were approved in the last years by FDA for AD treatment. Indeed, a progressive loss of cholinergic innervation in different CNS areas occurs in the pathology,[112] and thus a decreased cholinergic transmission is observed (Figure 3). Therefore, the inhibition of these enzymes allows an increased acetylcholine availability in the brain regions and a decreased deposition of Aβ.[113] Since the AChE levels in the brain areas are associated with progression of the

AD disease, likely limiting the therapeutic utility of this class of drugs, BChE levels in the disease is markedly high compared to the levels recorded in the healthy subjects. Thus, the inhibition of BChE represents an innovative therapeutic approach for AD.[114,115,116]

Although a wide structural heterogenicity can be observed among the AChE/BChE inhibitors, there are some specific molecular determinants that allow the identification of the basic requirements for inhibiting the two enzymes isoforms: a H-bond acceptor, an aromatic ring, a positive ionizable group, an hydrophobic portion, and the presence of tertiary amino groups, that can be protonated under physiological conditions.

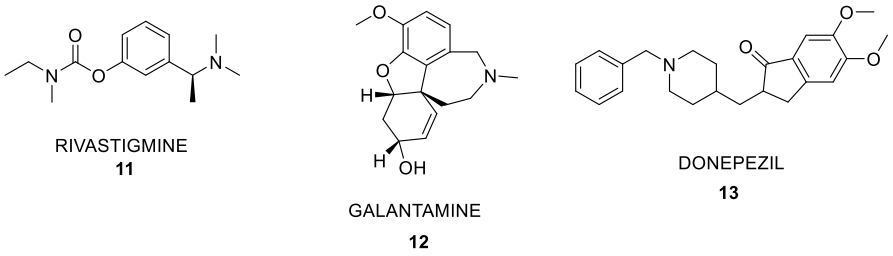

Figure 3. AChE inhibitors approved by FDA for AD treatment.

The idea of developing multitarget agents useful in AD treatment starting from CB2R agonists relied on the observation that some cannabinoids, such as JWH-015 (**8**, Figure 1), were able to induce the removal of β-amyloid plaques[117] and that THC[118] and some cannabinoid agonists, such as JWH-015 (**8**) and WIN 55,212-2 (**10**) were able to inhibit AChE *in vitro.*[26] With this in mind, Paez and colleagues proposed the first multitarget strategy for AD treatment based on the design of dual compounds with mixed CB2R/cholinesterase profiles.[26] They tested several cannabinoid ligands for their activity as AChE/BChE inhibitors and[26] performed rational structural modifications starting from the core of the CB2R agonist JWH-015 (Figure 1). In particular, by applying the concept of bioisosterism and by adding an aromatic ether, suitable for CB2R interaction (Figure 4),[27] they developed a series of indazoles and the best results were shown by compounds **14** and **15,** whose 2D structures are reported in Figure 4.

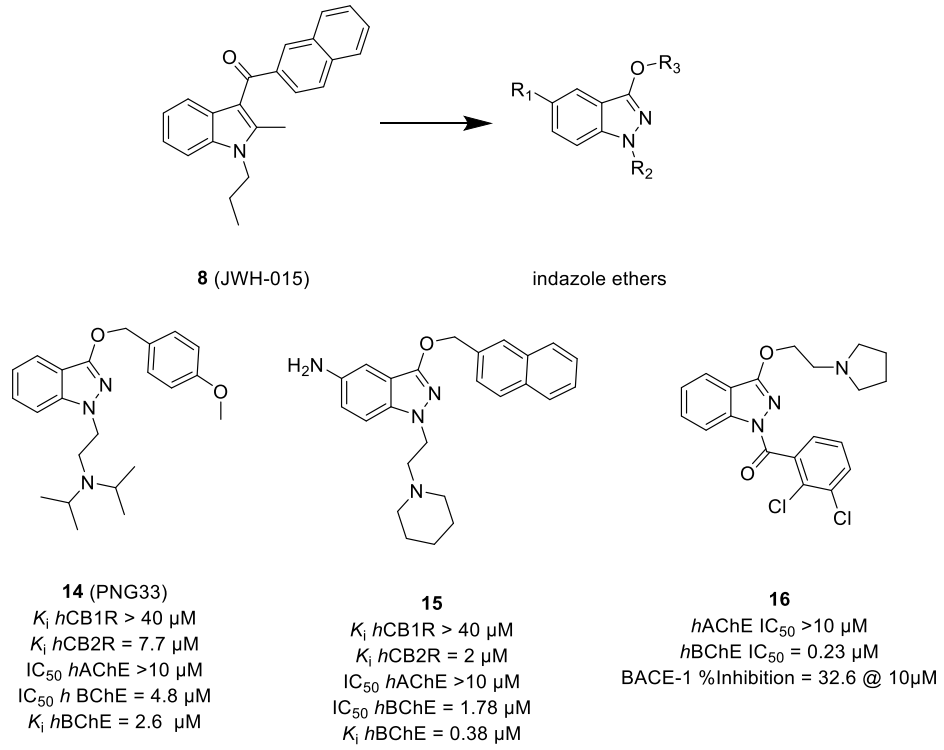

Figure 4: 2D sketch of the JWH-015 structural modifications proposed by Gonzalez-Naranjo and colleagues[27] for designing dual CB2R/ChE indazole compounds ligands, **14** and **15,** and CB2R/ChE/BACE-1 indazolyl ketones **16.**[28]

More recently, the same authors designed a series of new compounds having an indazolylketone as scaffold and showing a multitarget profile *vs* CB2R and cholinesterase. They started from 1-(2-di-*i*-propylaminoethyl)-3-(4-methoxybenzyloxy)indazole **14**[28] that could be considered an interesting starting point for the hit-to-lead development of BChE inhibitors and CB2R agonists. Structural modifications on the indazole led to obtain a family of dual CB2R agonists/BChE inhibitors with high selectivity, potency, and good biological profile. In this study, the multitarget effect was also evaluated against beta secretase 1 (BACE-1), the cleavage enzyme of beta-site amyloid precursor proteins (a key enzyme in the abnormal production of amyloid-beta protein which leads to fibrillary aggregation toxic to neurons). Based on pharmacological studies conducted *in vitro* and *ex vivo*, it

was observed that two of these compounds showed a CB2R agonist profile (isolated tissue assay) as well as a BChE and BACE-1 inhibitory profile.[28] Replacing the alkyl chain with an amide moiety maximizes the CB2R binding affinity by allowing the formation of new interactions. These indazolylketone derivatives can be considered as two- or three-target cannabinoids: compound **16** (Figure 4), indeed, acts as selective CB2R full agonist, in an isolated tissue assay, and, at the same time, as BChE and BACE-1 inhibitor. Its effect was also tested on Alzheimer's cell models and the obtained results put forward this compound as a promising agent for AD treatment. [28]

Very recently, 2-benzofuran-derived compounds, whose general scaffold is presented in Figure 6, were evaluated as potential cholinesterase inhibitors and CB2R ligands.[119] Montanari and colleagues identified some benzofuran analogues endowed with promising anti BChE activity and anti-aggregation properties towards beta amyloid plaques, but an undesired CB1R affinity. Starting from this promising scaffold and in order to direct the multitarget spectrum of action towards CB2R, they designed an original class of compounds as a result of different modifications on the central skeleton. In the most active compounds, the alkoxy *N*-methyl-*N*-benzylamine side chain was removed and substituted by a methoxy group, and the amines were directly linked to the benzophenone ring. Compound **17** showed inhibitory activity on BChE in the micromolar range, and CB2R selectivity over CB1R. Compound **18** is the most powerful CB2R ligand of the class, probably as a consequence of the nitrogen methylation. Compound **17** presents a broad spectrum of action, in fact it inhibits BChE, acts on CB2R with high selectivity over CB1R, shows a neuroprotective action and could be considered a promising MTDL, while compound **18** proved to be a potent CB2 inverse agonist and exerts an interesting multifunctional action by combining anti-inflammatory and neuroprotective effects.[119]

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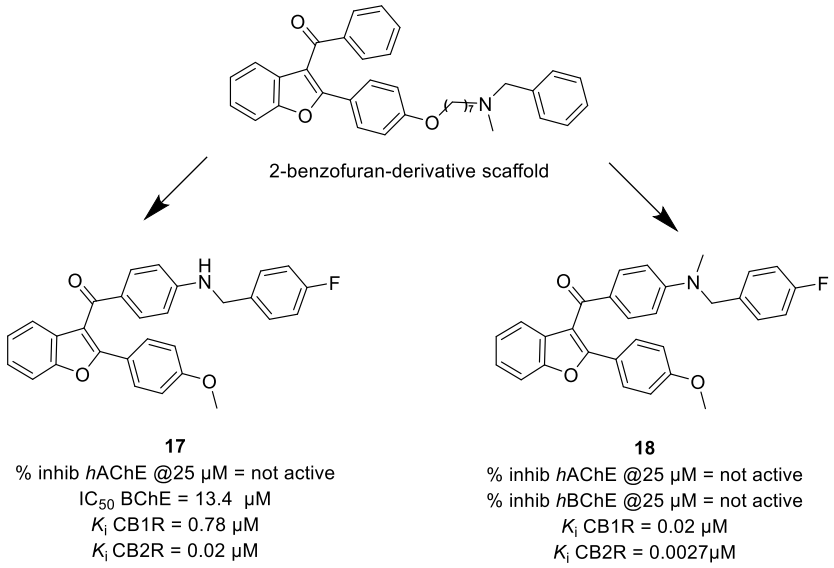

Figura 5. Dual CB2R/BChE benzofuran derivatives proposed by Montinari and collegues.[119]

In 2008, Astra Zeneca developed a very interesting and selective CB2R agonist (compound **20**, Figure 7).[120] On the basis of the pharmacophoric model of BChE inhibitors and of the results showed by the already reported indazole **14**, Dolles and colleagues in 2016 measured the ability of compound **20** to inhibit choline esterases finding interesting pharmacodynamic properties towards human CB2R (*h*CB2R) and equine BChE (*eq*BChE) (Figure 6).

Commentato [C5]: E' il composto 19? Figura 6? Altrimenti c'è un problema di corrispondenza tra figura, composto e riferimento

Commentato [C6]: Forse compound 19.

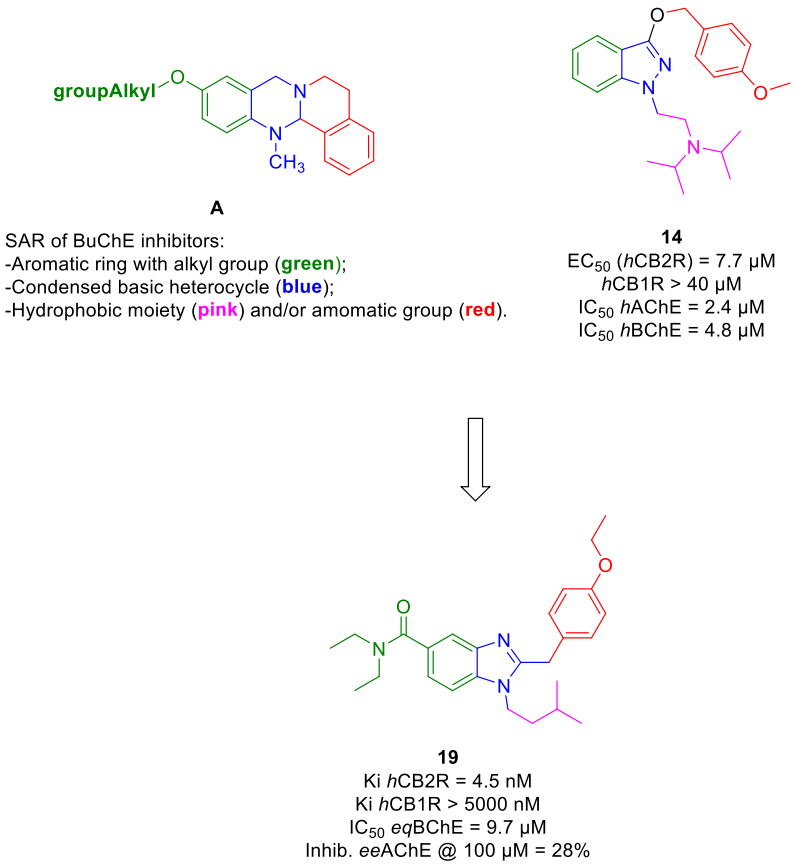

Figure 6. Pharmacophore model of BChE inhibitors and CB2R compound **14** (top); Benzimidazole derivative **19** proposed by Astra Zeneca[120] as *h*CB2R agonist and studied by Dolles and co-workers as BchE/AchE inhibitor (bottom).[29]

Starting from compound **19**, Dolles and co-workers proposed a second generation of dual CB2R/BChE benzimidazole derivatives by performing four structural changes: i) the replacement of the diethylamide function with a hydrogen atom, to confirm the need of this fragment for the interactions with CB2R and BChE; ii) the introduction of a basic centre on the hydrophobic alkyl chain; iii) the modifications of the linker connecting the imidazole moiety and the phenyl ring; iv) the modification of the heterocycle core.[29] The results clearly indicated that the presence of the diethylamide function is pivotal to ensure both the affinity *vs* *h*CB2R and the inhibition of

*eq*BChE.[29] For this reason, the authors focused their attention on substitutions in other portions of the molecule. The performed changes aimed to increase the basicity of the final compound through the insertion of amino groups to improve the interactions with the *eq*BChE active site. Interesting results came from the introduction of a piperidine ring on the alkyl chain obtaining compound **21**. Compound **19**, **20** and **21** presented moderate affinity towards *h*CB2R, but an interesting *eq*BChE inhibition (Figure 8). Other modifications led to 2-aminobenzimidazole compound **22** that showed a moderate affinity *vs h*CB2R but, also in this case, a good inhibitory effect on *eq*BChE.[29]

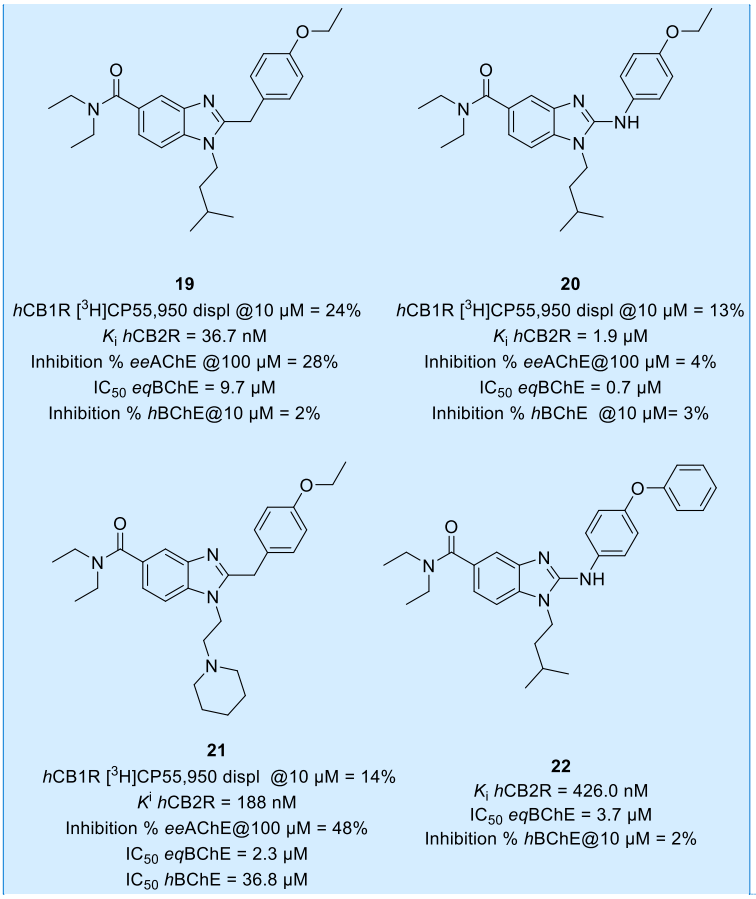

Figura 7. Dual CB2R/BChE benzimidazole derivatives proposed by Dolles and colleagues: second generation.[29]

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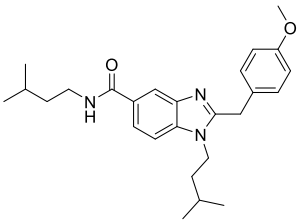
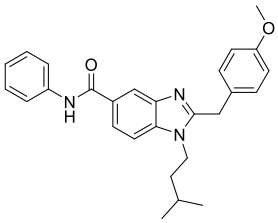
However, the excellent results were not recapitulated in a later study in human BChE.[29] A third generation of analogues of compound **19** was studied by performing additional structural changes such as the: i) diethylamide substitution in position 5 of the main scaffold; ii) substituent modification in position 5 (reverse amide, nitro group and primary amine); iii) merging between the most interesting CB2R and BChE pharmacophoric fragments (Figure 8).

Starting from compound **19**, the introduction in 5-position of different amides determined a decrease of *h*CB2R affinity but an interesting increase in *h*BChE inhibition. The 1-piperidinyl amide (**25)** determines an inhibitory activity on *h*BChE and higher CB2R affinity compared to the linear analogues. The anilineamide **24** showed the lowest CB2R affinity of this series (Figure 8).

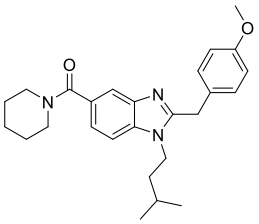
Different amide structures in 5-position of the benzimidazole scaffold



23
K_i *h*CB2R = 763.3 nM
IC_{50} *h*BChE = 1.6 µM

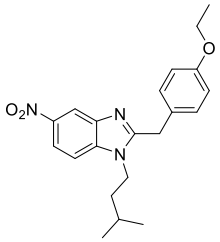


24
K_i *h*CB2R = 14.4 µM
IC_{50} *h*BChE = 1.2 µM

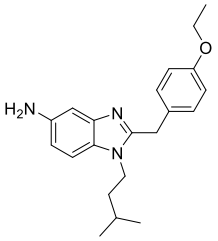


25
K_i *h*CB2R = 384.5 nM
IC_{50} *h*BChE = 5.7 µM

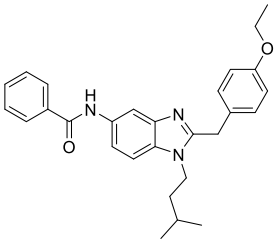
Different substituents in 5-position of the benzimidazole scaffold



26
K_i CB2R = 961.8 nM
Inhibition *h*BChE = 12% @50µM

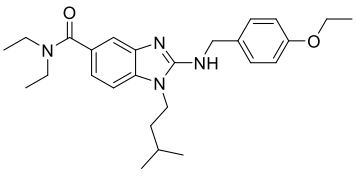


27
K_i CB2R = 21 µM
Inhibition *h*BChE = 39% @100µM

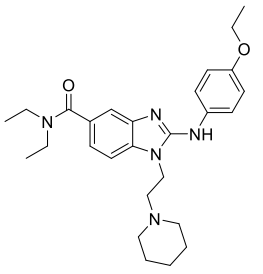


28
K_i CB2R = 4.3 µM
Inhibition *h*BChE = 25% @10µM

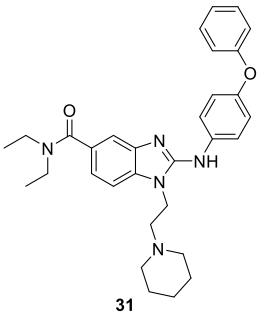
Merging between the most interesting CB2R and BChE pharmacophoric fragments



29
K_i *h*CB2R = 353.4 nM
Inhibition %*h*BuChE = 4% @10µM



30
K_i *h*CB2R = 10.4 µM
IC_{50} BuChE = 53.4 µM



31
K_i hCB2R = 8.9 µM
Inhibition %*h*BuChE = 4% @10µM

Figure 8. Dual CB2R/BChE benzimidazole derivatives proposed by Dolles and collegues, third generation.[30]

In Figure 8, modifications in 5-position of compounds **19** were also evaluated. The introduction of electron-withdrawing/donating substituents, in place of the diethyl amide group, determined a complete loss of the activity/affinity *vs* both targets. Interestingly, compound **28**, with an inverse anilineamide, maintained CB2R affinity but lost the *h*BChE inhibition.[30] By merging structures **20**, **21** and **22,** Dolles and colleagues developed the compounds illustrated in Figure 8. The introduction in compound **21** of an additional methylene between the amino group and the phenyl ring (compound **29**) determined an increase of CB2R affinity but no *h*BChE inhibition. Compound **30** derives from merging compounds **21** (*p*-ethoxy phenyl core) and **22** (ethylene piperidinyl core), while compound **31** results from **22** (*p*-phenoxy phenyl core) and **23**, but an improvement in terms of pharmacological profile was not observed.

The authors also explored changes of the length of ethylene pyridinyl moiety of compound **21** without obtaining interesting results in terms of *h*CB2R affinity, selectivity towards *h*CB1R and selective BChE/AChE inhibition.

In conclusion the strategy followed by the authors could be considered as very representative for a MTDL design. Indeed, the developed compounds showed balanced affinities/activities towards the selected targets and were proved to improve cognition *in-vivo*.[29]

In a more recent paper, Scheiner and colleagues reported the synthesis of a series of hybrid compounds derived from the combination of tacrine (a cholinesterase inhibitor (ChE)) and compound **19** through different spacers.[31]

The amide group and the N1 of the imidazole core are the most appropriate groups to be connected by the linker hence obtaining two series of ligands. The hybrids of both series resulted CB2R agonists and AChE/BChE inhibitors (ChE) (Figure 9).

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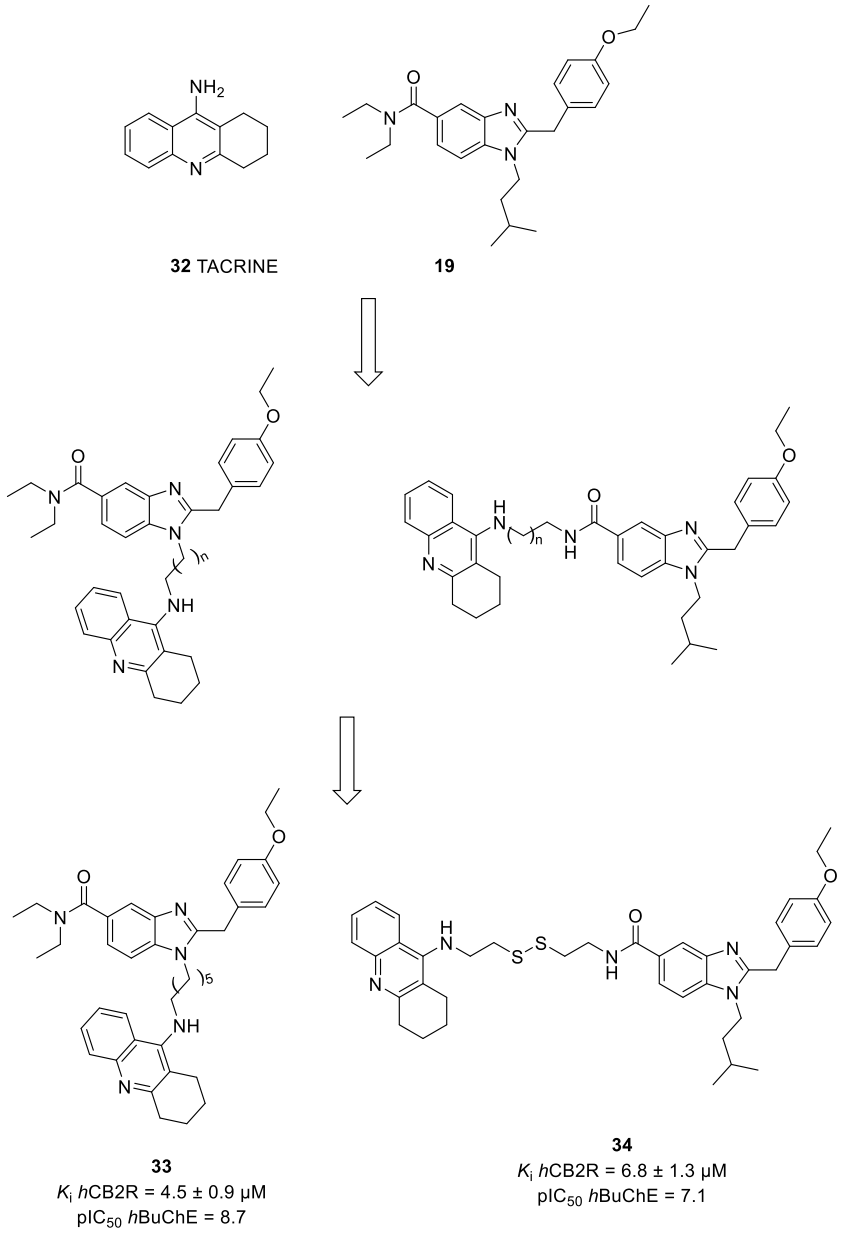

Figure 9. General structure of hybrid compounds derived from the combination of tacrine and compound **20**[31] reported by Scheiner and collegues.[31]

An additional strategy consisted in the insertion of an additional linker such as a disulfide bridge with the aim to improve the pharmacokinetic profile (**34**). All these hybrids have maintained their behaviour as CB2R agonist and the related immunomodulatory effect showed by compound **19**. Compounds **33** and **34** were able to reduce the Beta-amyloid plaques concentration with neuroprotective effect despite a not so high potency as ChE inhibitors and CB2R agonists.[31]

■ DUAL CB2R/HDAC FOR CANCER AND NEURODEGENERATION THERAPY

Histone acetylation is crucial for the transcriptional activity.[121] The balance between acetylation and deacetylation is the principal epigenetic control of genetic transcription and it is mediated by two important classes of enzymes: Histone acetyl transferase (HAT) and Histone Deacetylase (HDAC).[122] The loss of acetylating agents in combination with increased HDAC activities is one of the most frequent epigenetic anomalies occurring in cancer. It was reported[123] that the repression of onco-suppressor genes is one of the principal causes of carcinogenesis and tumor progression, and among all the epigenetic modulators, HDAC enzymes have a pivotal role in this context. At the beginning of this process, HDAC enzymes restrain genes devoted to the check of cells growth and this determines an uncontrolled cellular proliferation, loss of differentiation and apoptosis inhibition. Moreover, in the cancer progression, HDAC enzymes silence genes appointed to check cellular adhesion, migration and invasion.[124] Recently, HDAC involvement in neurodegenerative disease was also reported and reviewed. [56]

For all these reasons, HDAC inhibitors (HDACi) represent an interesting option in cancer research. They are able to activate pathways involved in apoptosis and also to modulate the expression of non -histone proteins such as DNA repair enzymes, transcription factors and nuclear regulators.[56] Each HDAC enzyme presents a common deacetylation domain constituted by 390 aminoacids. The catalytic pocket is strict and cylindrical presenting a central Zn^{2+} atom that, coordinated by a network of Histidines and Aspartates, performs its catalytic activity.[125-129]

Some representative HDACi are illustrated in figure 15. As expected, all of them present a typical Zn^{2+} binding group (ZBG), which is usually a hydroxamic acid or a 2-aminoanilide; a linker; and a hydrophobic cap group able to interact with the enzyme surface.

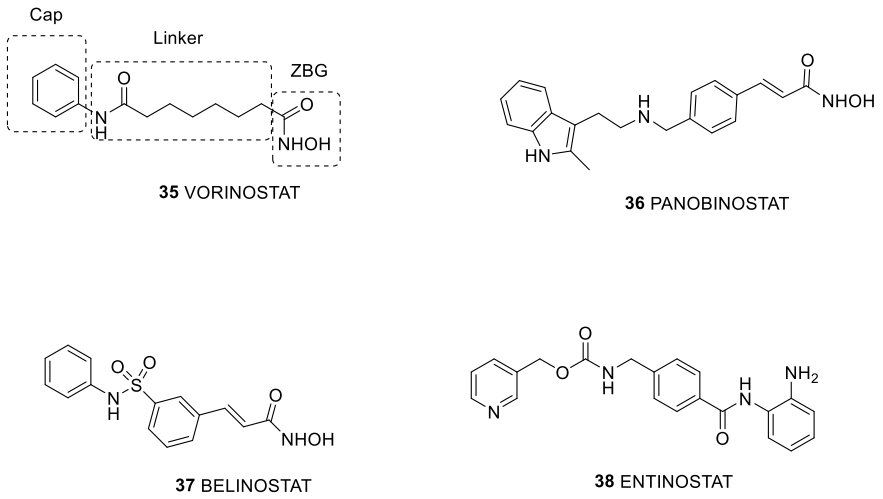

Figure 10 Representative HDAC inhibitors.

The combination of HDACi with other drugs, able to hit other antitumoral targets, can induce a synergic positive effect in the therapy of cancer, as recently reported.[130, 131]

The successful use of HDACi in the same cancer cell lines where CB2R are overexpressed as well as their anti-inflammatory properties in the same pathologies where CB2R is involved[132–139] suggest the development of dual CB2R/HDAC as a valuable strategy for cancer and neurodegeneration therapy. Our idea is also supported by many recent works proposing chimeric HDACi as a winning strategy for cancer.[140, 141]

Based on this evidence and inspired by Stazi *et al.*,[140] reporting HDACi hybrids obtained through a molecular hybridization approach, herein we propose that linking a known CB2R scaffold to a proper ZBG group might be a valuable strategy to design compounds acting as dual CB2R/HDAC.

For example, Panobinostat (**36**, figure 10), a well-known HDACi, and many CB2R agonists (*e.g.* compound JWH-015, **8**) share an indole group, and could be considered an interesting starting point. Noteworthy, it was already evidenced the versatile ability of the indole group to interact with diverse targets and to confer drug-like properties.[142]

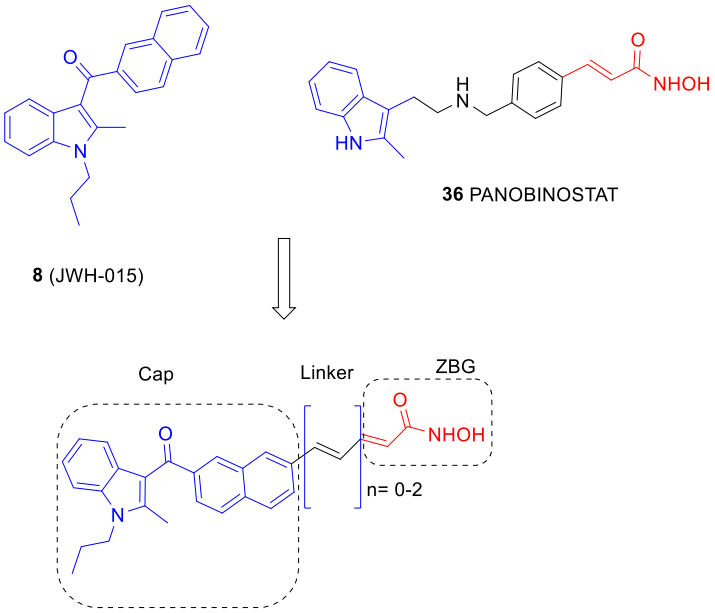

Figure 11. Proposed dual CB2R/HDACi obtained by merging compounds **8** and Panobinostat.

Another strategy may consist in the introduction of CB2R pharmacophoric fragments on HDACi scaffolds (merging approach). In this fashion, a problem could arise from the introduction on HDACi structure of the bulky cycloalifatic carboxamide group (usually an adamantyl carboxamide) very useful to recover CB2R affinity. Mercifully, the work of Cincinelli and coworkers,[143] very recently reported the synthesis of HDACi presenting the large hydrofobic adamantyl group and showing a good efficacy as antiproliferative and proapoptotic agents.

Interestingly, also Gopalan and colleagues reported the discovery of adamantane based highly potent HDACi,[144] thus confirming that the presence of a bulky adamantyl core, although very bulky, is compatible with an inhibitory activity towards HDAC.

On this basis, 2-aminoanilide represents another HDAC scaffold that can be proposed as starting point for satisfying CB2R pharmacophore needs.

In particular, the structure of entinostat (**38**, Figure 12) could represent a suitable starting scaffold for the introduction of CB2R pharmacophoric substituents such as a cycloaliphatic (also the adamantly) carboxamide and the essential aliphatic chain.

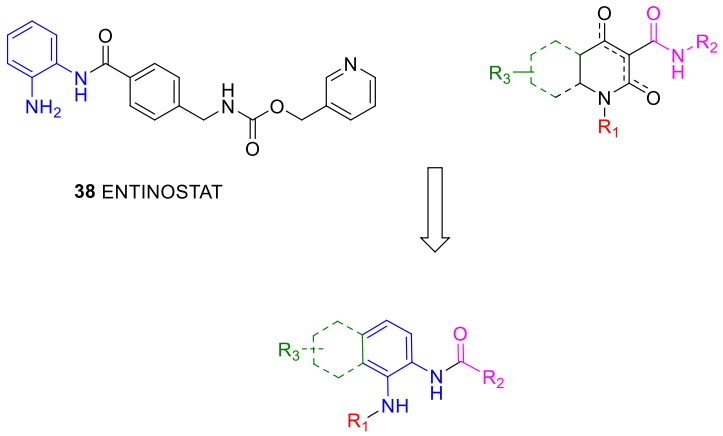

Figure 12. Proposed strategy to design dual CB2R/HDACi: merging approach involving the structure of entinostat and the CB2R pharmacophore**.**

As reported in the following sections, all these suggestions, based on the inspection of the available literature, may represent a promising starting point for setting up rational strategies based on an effective interplay between experimental and computational approaches.[33]

■ DUAL CB2R/SIGMA FOR CANCER AND NEURODEGENERATION THERAPY

Sigma-1 and sigma-2 receptors are other targets that we propose as promising for the development of dual drugs, being both involved in cancer and neurodegeneration. Sigma-1 receptor is a chaperone protein expressed in the central nervous system (CNS),[145] where it plays a role in neurodegeneration, and in peripheral organs and in immune and endocrine tissues,[145] where the protein is mainly studied for its involvement in cancer disease. Because of its chaperone nature, sigma-1 receptor is usually associated with binding immunoglobulin protein (BiP) in a resting state

on the surface of endoplasmic reticulum (ER), in an area known as the mitochondrial associated ER membrane (MAM). After activation by ligands or Ca^{2+} depletion from ER, sigma-1 receptor dissociates from BiP and binds to Inositol 1,4,5-trisphosphate receptor type 3 (IP3R3) in order to increase Ca^{2+} fluxes from ER to mitochondria and to regulate IRE1-dependent pathway, which neutralizes ER stress. Among the activities associated with sigma-1 receptor, worthy of note are also the antiapoptotic and antioxidant ones resulting from the interaction with Bcl-2, and Nrf2.[146] Several ligands have been developed as modulators of sigma-1 receptor, allowing the identification of a commonly accepted pharmacophoric model that works well irrespective of the ligand activity (antagonism and agonism): an amine site flanked by two hydrophobic regions.[147]

Sigma-2 receptor is less known and understood. Through the years, diverse identities have been attributed to this still little-known receptor. After the histone hypothesis[148,149] sigma-2 receptor was proposed as the progesterone receptor membrane component 1 (PGRMC1)[150] and recently as TMEM97.[151] The sigma-1 subtype is strictly connected to several neurodegenerative diseases (such as Amyotrophic Lateral Sclerosis (ALS), [152-156] Parkinson's disease (PD), [157-161] Huntington's disease (HD),[162,163] Alzheimer's disease (AD)[164,165] with sigma-1 receptor agonists that improve neurotransmission and exert anti-neurodegenerative effects.[161-167,156] The sigma-2 subtype has only recently been associated with AD, as a class of sigma-2 receptor antagonists inhibit the binding of Aβ oligomers to their neuronal binding sites, reducing the neurotoxic effects.[168,169] Importantly, one of these molecules CT1812 (Elayta^{TM}) has entered phase 2 clinical trials for early AD treatment.

As for its involvement in cancer, sigma-1 receptor is expressed in many human tumor cell lines,[39] where it influences ion channels (highly expressed in cancer cells)[170] and regulates essential processes for tumor expansion and cell survival such as mitosis and apoptosis.[171] Therefore, sigma-1 receptor antagonists such as rimcazole, IPAG, reduced haloperidol, BD-1047, BD-1063[172] and PB212[173] can inhibit tumor cell survival.

Sigma-2 receptor has been widely studied in the oncology field, where the effect on cell proliferation of its modulators, through diverse mechanisms, mainly relies on the modulation of ER

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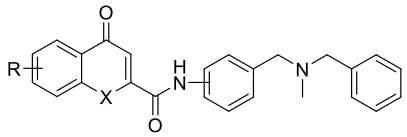
stress through the control of Ca^{2+} release,[174–176] the increase in ROS[177] and the mitochondrial superoxide production.[178]

According to the MTDL strategy, these pieces of evidence prompt us to connect the antineurodegenerative and anticancer activities of CB2Rs with the properties of sigma receptors. We believe that hitting these targets at the same time could provide a successful strategy to modulate, in a synergistic manner, diverse biological pathways underlying cancer and neurodegeneration. Dual CB2R/sigma have never been synthesized, but the exploitation of scaffolds with proven affinity for both the receptors classes, according to the corresponding pharmacophores, could represent a valuable starting point.

As an example, the herein reported 4-Quinolone class (Figure 13), which is proved to have high CB2R affinity, was used by Estrada-Valencia and colleagues to design MTDLs able to bind sigma-1 and other targets involved in AD, such as AChE, MAOs, BACE-1 and LOX-5.[179]



X = O or NH

Figure 13. General structure of Estrada-Valencia's ligands.

Another example is represented by the 2-Quinolone class, which has been explored by Mugnaini and colleagues to develop CB2R ligands with anticancer activity,[180] while Weber and colleagues produced 3,4-dihydro-2-quinolones as sigma-1 receptor antagonists with antinociceptive action (Figure 14).[181] These studies provide evidence of the common scaffolds which can be employed in order to obtain dual CB2R/sigma ligands. Modification of these scaffolds with the insertion of a basic moiety on the quinolinone nucleus for the Mugnaini's ligand **39**, or with a bulky alkyl amide for the Weber's ligand **40** could correspond to an overlap of the CB2R and sigma-1 receptor pharmacophore leading to the desired dual ligands. Based on this evidence, structure-based and

ligand-based approaches, as reported in the following section, can be employed to select the appropriate positions for convenient structural changes, guiding also the selection of other scaffolds different from those herein proposed.

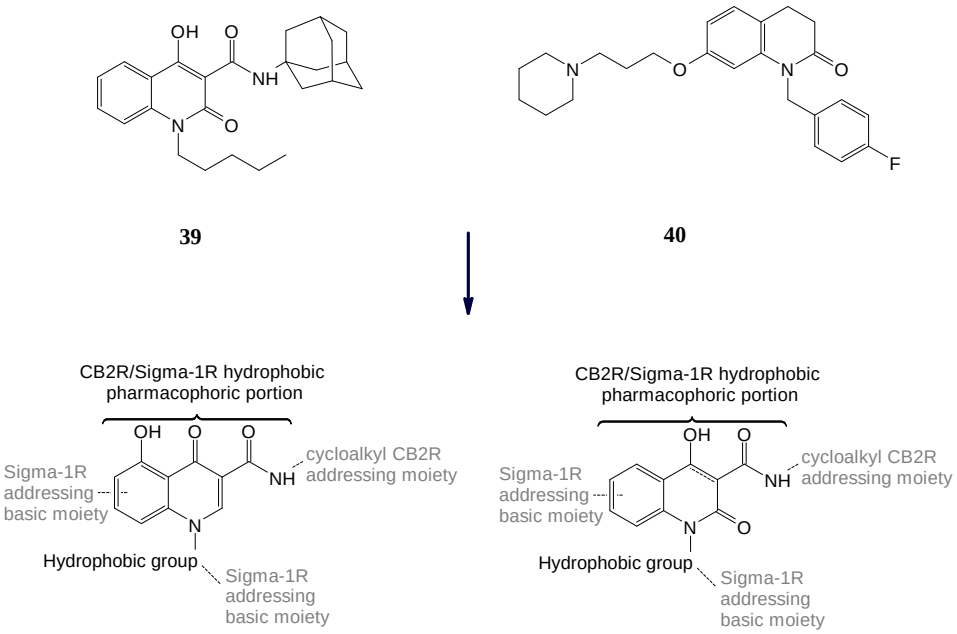


Figure 14. Mugnaini's ligand **39**, on the left; Weber's ligand **40** on the right; Proposed strategy to design dual CB2R/SIGMA: an outline of possible modifications is reported on the bottom**.**

The same strategy could also be applied for the development of dual CB2R agonists and sigma-2 receptor ligands, with similar therapeutic perspectives outlined for the sigma-1 subtype. However, despite the fact that the two sigma subtypes share similar pharmacophoric elements, the lack of the sigma-2 receptor crystal structure makes this task more difficult, hampering the application of *in-silico* structure-based approaches.

■ IN-SILICO APPROACHES FOR THE IDENTIFICATION OF DUAL CB2R/HDAC AND CB2R/SIGMA COMPOUNDS.

Although the first chemicals able to act on multiple targets were discovered by serendipity, MTDLs are today designed by means of rational approaches. As extensively reviewed in the last years,[33,109,182] employing state-of-the-art computational strategies is mandatory to properly approach such a difficult issue saving money and time. More specifically, molecular modelling (MM) is in the spotlight (i) to select promising target combinations at the early stages of a multi-target drug discovery project; (ii) to identify initial hits acting on multiple targets; (iii) to optimize the multi-target activity during the "hit-to-lead" and "lead-optimization" phases. A fundamental prerequisite for employing MM approaches is the knowledge of molecules acting on the targets of interest (*i.e.* ligand-based rational design - LBRD) or of the three dimensional information of the target structures (*i.e.* structure-based rational design - SBRD). As described below, such conditions are totally met when CB2R, HDAC and sigma-1 receptor are taken into account while, on the contrary, the available information regarding sigma-2 receptor is still too poor to apply reliable MM approaches, although we are confident that this limit will be overcome in the next future due to the increasing importance of this target. In this section, we will outline viable computational approaches to guide the identification and design of dual CB2R/HDAC and CB2R/sigma-1, with the aim of providing valuable preliminary information for medicinal chemists interested in the field. In particular, a special attention will be devoted to emerging strategies based on the application of machine learning techniques.

Structure- and ligand-based rational design

CB2R/HDAC and CB2R/sigma-1 represent promising target associations not only from a therapeutic but also from a methodological point of view. The high amount of data, today available in freely accessible data resources such as Protein Data Bank,[183] containing information about experimentally-determined structures of proteins, and CHEMBL,[184] containing a curated database of bioactive molecules, constitutes an ideal playground for planning reliable SBRD and LBRD strategies. Noteworthy, in the last years the absence of an atomic-resolution structure of CB2R has represented the main hindrance to the application of SBRD strategies on this target, although

examples of research efforts in this direction, based on the development and employment of homology models, are available in the literature.[185–187] Such a limitation has been overcome only in 2019 when Li *et al*. deposited the first x-ray structure of human CB2R in the Protein Data Bank (PDB ID: 5ZTY[188]), thus making reliable the application of SBRD approaches, as we showed in a recently published paper.[20] Even more interestingly, Hua *et al*. published in 2020 the first crystal structure of an agonist bound human CB2R (PDB ID: 6KPC[189]), thus providing unprecedented structural information for rationally designing CB2R agonists. As far as HDACs are concerned, several 3D structures of different human isoforms are available in the Protein Data Bank. Examples are given by several X-ray structures, in complex with inhibitors, of HDAC2 (PDB ID 3MAX,[190] 5IX0,[191] 5IWG,[191] 6WBZ,[192] 6WBW,[192] 6G3O,[193] 4LXZ[194] and 4LY1[194]), HDAC4 (PDB ID: 2VQQ,[195] 2VQM,[195] 2VQJ,[195] 5A2S,[196] 4CBY,[196] 4CBT[196] and 6FYZ[197]), HDAC7 (PDB ID: 3ZNR,[198] 3ZNS,[198]3C0Z[199] and 3C10[199]) and HDAC8 (PDB ID: 1VKG,[200] 1T64,[200] 5D1B,[201] 1T69,[200] 1T67[200] and 2V5X[202]). Some of these structures have been successfully employed in the last years to identify, for instance, HDAC4,[203] HDAC8,[204] dual MMP-2/HDAC-8[205] and dual MMP2/HDAC-6[206] inhibitors by means of SBRD strategies. Valuable structural information is today available also for sigma-1 receptor. In 2016 Schmidt *et al*. published on Nature the first crystal structures of its human form in complex with PD144418 (PDB ID: 5HK1[207]) and 4-IBP (PDB ID: 5HK2[207]) while, two years later, the same research group made available the crystal structures of human sigma-1 in complex with haloperidol (PDB ID: 6DJZ[208]) NE-100 (PDB ID: 6DK0[208]) and (+)pentazocine (PDB ID: 6DK1[208]). All these receptor-ligand complexes show a common ligand binding mode where an interaction between a basic amine and Glu172 seems to be crucial for molecular recognition. Importantly, Greeenfield at al. have recently identified high affinity sigma-1 receptor ligands by employing a docking based virtual screening (VS).[209] These results allowed them to suggest this receptor as an ideal candidate for SBRD strategies. All these pieces of evidence, taken as whole, provide a breeding ground to guide the identification and design of CB2R/HDAC and CB2R/sigma-1 MTDLs by means of SBRD strategies. Binding site similarity

analysis can be performed in order to select, for instance, which HDAC structure (and therefore isoform) is better suited for designing dual CB2R/HDAC compounds by comparing the available CB2R and HDAC pockets. Cavities can be characterized by means of several methodologies[210] based, for instance, on quadruplet fingerprints applied to molecular interaction fields[211] or Principal Component Analysis (PCA) applied on properties such as size, polarity and charge.[212] Hierarchical computational platforms for multilayer VS of large chemical libraries can be developed based on molecular shape similarity, structure-based pharmacophore models and molecular docking simulations.[213] VS campaigns can be independently performed on CB2R, HDAC and sigma-1 and promising hits can be identified from those compounds located at the top of the ranked lists. Molecular docking simulations can be also employed to investigate the proposed scaffolds based on molecular hybridization (Figures 11, 12 and 14) and suggest the most promising structural changes to be considered for designing the first MTDLs acting on these targets. Due to the currently available data, CB2R/HDAC and CB2R/sigma-1 MTDLs might be also identified by means of LBDD approaches based, for instance, on the development and application of Quantitative Structure-Activity Relationship (QSAR) models or similarity-based algorithms for target prediction.[214] Examples of successful application of the so-called "multi-target QSAR" are available in the literature[215–217] and have been recently reviewed by Abdolmaleki *et al*.[218] Noteworthy, ChEMBL (version 27.1) contains curated experimental data concerning 3989, 1678 and 2274 compounds provided with high affinity (IC_{50} or K_i values $\leq$ 1μM) towards human CB2R, HDAC and sigma-1 receptor respectively. Figure 15 shows the projection of these compounds into the top two principal components (PCs) obtained from 11 topological and 323 physicochemical descriptors computed for each ligand using Canvas,[219] available in the Schrodinger suite, as software. The plot clearly indicates the presence of a common chemical space covered by CB2R and HDAC (Figure 15A) as well as CB2R and sigma-1 (Figure 15B) binders, thus supporting the idea, based on the inspection of the proposed pharmacophores, that these target combinations are promising for

designing MTDLs. Notice that in a chemical space representation the closeness of the represented points reflects the structural and/or property similarity of the corresponding compounds.

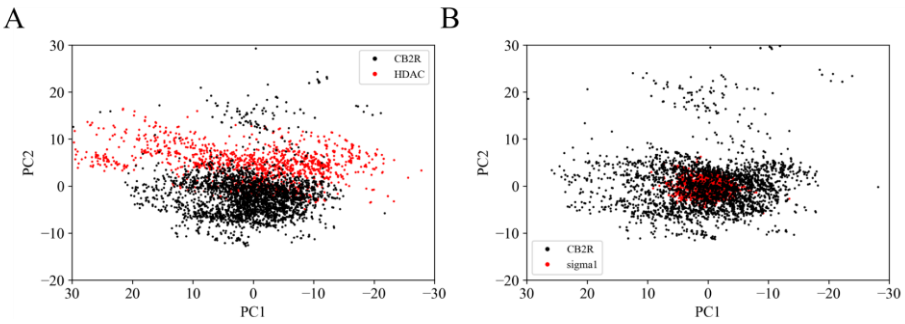


Figure 15. Projection of A) CB2R (black points) and HDAC (red points) and B) CB2R (black points) and sigma-1 (red points) high affinity (IC_{50} or $K_i \leq 1\mu M$) compounds into the top two PCs obtained from 11 topological and 323 physicochemical descriptors. Notice that PC1 and PC2 are plotted accounting for: A) 90% (CB2R/HDAC) and B) 87% of the total variance.

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Last but not least, the available public data can be implemented to identify CB2R/HDAC and CB2R/sigma-1 MTDLs by integrating SBDD and LBDD approaches in a single technique.[220] Indeed, combining the information on the protein structures with that on the physicochemical and biological properties of bound ligands can strongly enhance the success rate in multi-target drug discovery programs. Noteworthy, this strategy proved effective for the identification of a first dual compound acting on Heat shock protein 90 (Hsp90) and tubulin[221] two important anticancer targets.

Machine-Learning-Assisted De Novo Design

Due to the available knowledge of ligands acting on CB2R, HDAC and sigma-1, machine learning can be also employed to design CB2R/HDAC and CB2R/sigma-1 MTDLs. Such approaches, widely used in several application domains such as image understanding, signal processing and matter engineering, are today emerging as particularly effective for *de-novo* design of compounds having desired properties, including biological activities towards specific targets of interest.[222]

The main aspect of these techniques is that they are able of learning a representation of a given physical or abstract phenomenon described by a functional relation y=f(x), i.e. a mapping function between the input variables or observations (for example, fingerprint representations of molecules) and an output variable that can be discrete or continuous such as an active/inactive label or the bioactivity value. The data take form of input/output pairs (x,y) called examples and collected during the observation of the phenomenon. In mathematical terms, *de novo design* aims to find an element x that is related to the value y: y = f (x). In molecular terms, x is a molecular structure from the chemical space and y is the descriptor of x computed by function f. The problem begins to learn a representation of *f* by using a training set composed of N examples (x,y). This form of learning is known as supervised learning, as there is a teacher providing the value y for each input x in the training phase. There is another form of learning that is unsupervised and relevant for *de-novo* drug design called density estimation.[223] We can imagine the chemical space of compounds as a compact continuous space that includes a probability density p, the goal is to learn this probability density in order to sample from it new observations. Recently, this difficult problem has been tackled under a different and appealing perspective. Instead of estimating the density *p* and then sampling *p* for obtaining new observations, we can directly generate new observations. This class of techniques, known as generative models, use deep neural networks for representing the probability density functions. Probabilistic models based on neural networks are computationally scalable since they use stochastic gradient-based optimization which allows scaling to large models and large data sets.[224, 225] The simplest form of generative model for *de-novo* drug design is described in Gupta *et al*. 2018.[225] The authors explore the vast chemical space for compounds which may not have been synthesized before by using a generative deep learning model based on Recurrent Neural Networks (RNNs), the network of choice for tasks involving sequential inputs. Indeed, the authors used Simplified Molecular Input Line Entry Specification (SMILE) representation. RNNs process an input sequence one element at a time, maintaining in their hidden units a state vector that implicitly contains information about the history of all the past elements of the sequence. Their recurrent

network is composed of two layers of Long Short-Term Memory (LSTM) cells first introduced by Hochreiter and Schmidhuber in 1997[226] for solving the gradient vanishing problem affecting the vanilla RNNs. Trained with backpropagation through time, their model was able to generate valid SMILE strings with high accuracy, structurally similar to drugs with known activities against particular targets. In other words, by training RNNs on datasets containing compounds with high affinity towards human CB2R, HDAC and sigma-1 receptor, one could generate compounds which can be considered as ideal MTDLs candidates. More sophisticated deep generative models, well suited for multi target drug *de-novo* design, make use of autoencoders for converting molecular discrete representations of variable length to continuous, fixed length vectors belonging to a latent space of r.v. in which to generate novel chemical compounds by performing simple algebraic operations such as interpolating between molecules. Variational autoencoders (VAE)[227] are generative models composed of an encoder which maps SMILES representations of molecules into a latent space and of a decoder which converts latent vectors to the original input SMILES. Both encoder and decoder are implemented by using multilayer RNNs with LSTM units. The main aspect of VAE consists in learning the parameters of probability distribution of the latent space and in using these parameters for generating new latent vectors corresponding to novel compounds with given properties. In other terms, generating chemical structures by optimizing with respect to selected properties (such as activity towards more targets of interest) can be performed by optimizing a reward function in the continuous latent space. Gómez-Bombarelli *et al*. utilized Bayesian Optimization[227] to find points in the space corresponding to molecules with a desired property. Another approach for fine-tuning a generative model in order to design molecules with a specific property is Reinforcement Learning. In the case of *de-novo* design, Reinforcement Learning aims at learning how it is possible to optimize a reward in a given space. Olivecrone *et al*.[222] utilized this concept to modify the generative process of a pre-trained RNN to generate a set of molecules enriched of compounds with desirable properties. An appealing alternative to VAE are the Generative Adversarial Networks (GAN) adopted in Kudurin *et al*.[228] to identify new molecular

fingerprints with predefined anticancer properties. GANs are based on a minimax game. A generative model G (generator) is opposed to a discriminative model D (discriminator) that learns to determine whether a sample is drawn from the data distribution or from the distribution generated by G (model distribution). This model G is analogous to a group of counterfeiters, trying to produce counterfeit money, while the model D is analogous to the police, trying to detect the fake currency. During the competition, both the counterfeiters and the police try to improve their strategies until the counterfeits are indistinguishable from the true articles. So, D has to minimize the error rate in discriminating true from fake samples, and G has to maximize its ability to generate samples similar to the real data. Both D and G are described through multilayer perceptron and optimized through backpropagation. A characteristic of such generative models is that they do not explicitly represent the likelihood, yet they are able to generate samples from the desired distribution. GANs differ in how they generate molecules, but they all apply Reinforcement Learning to generate more molecules with structures that are active on both targets of interest. Successful generative models for the *de-novo* design of multi-target compounds are available in the literature. Of note is the paper by Winter *et al*.[229] By using a lighter weight heuristic optimization method termed Particle Swarm Optimization (PSO), the authors designed *de-novo* compounds with predicted multi-target activity towards the epidermal growth factor receptor (EGFR) and aspartyl protease b-site APP cleaving enzyme-1 (BACE1). Noteworthy, they applied PSO in a continuous chemical representation and used it to optimize molecules with respect to a multi-objective value function defined as a combination of multiple molecular properties. The model was able to generate compounds with optimized metabolic stability, predicted solubility, cell permeability, drug-likeness as well as a good synthetic accessibility. Another meaningful example is represented by a paper published while writing this perspective. In particular, Tan *et al*.[230] reported an automated deep learning workflow for the automatic design of MTDLs. The developed model was able to generate molecules with potent activities towards dopamine D_2, serotonin 5-HT_{1A} and 5-HT_{2A} receptors, a polypharmacology profile highly desirable to develop drugs with antipsychotic effects.

■ CONCLUSIONS

CB2R plays a crucial role in neurodegeneration and several types of tumours due to its involvement in the (neuro)inflammatory process and, recently, it has been successfully associated to other targets such as AChE and BchE for developing MTDLs with beneficial polyphamacology effects for the AD treatment. Starting from this evidence, in this paper we prompt the scientific community to consider new target associations, namely CB2R/HDAC and CB2R/sigma receptors, for developing new and promising MTDLs for cancer and neurodegeneration therapy. Such a suggestion is based on: i) an in-depth inspection of the available literature indicating the presence of common pharmacophoric elements; ii) the available public information concerning the 3D target structures as well as several known high affinity ligands. The paper is discussed in the perspective to provide a guide for medicinal chemists interested in this attractive opportunity and having experience in the fields of molecular hybridization, molecular modelling and/or machine learning guided de-novo design.

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■ ABBREVIATIONS USED

5-HT_{1A}, serotonin receptor subtype 1A; 5-HT_{2A}, serotonin receptor subtype 2A; μM, micromolar; Aβ, amyloid β-protein; AChE, acetylcholine esterase; AD, Alzheimer's disease; ADME, absorption, distribution, metabolism and excretion; ALS, amyotrophic lateral sclerosis; APP, amyloid-β precursor protein; BACE-1, beta-secretase; BChE, butyrylcholinesterase; Bcl-2, B-cell lymphoma 2; BiP, binding immunoglobulin protein; CB1R, cannabinoid receptor subtype 1; CB2R, cannabinoid receptor subtype 2; CNS, central nervous system; D_2, dopamine receptor subtype 2; D, discriminator; DNA, deoxyribonucleic acid; EC_{50}, half maximal effective concentration; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; FDA, Food and Drug Administration; G, generator; GAN, Generative Adversarial Networks; HAT, histone acetyl transferase; HD, Huntington's disease; HDAC, histone deacetylases; HDACi, HDAC inhibitors; Hsp90, Heat shock protein 90; i-Pr, isopropyl; IC_{50}, half-maximum inhibitory concentration; IP3R3, inositol 1,4,5-triphosphate receptor type 3; IRE1, inositol-requiring enzyme 1; Ki, inhibition constant; LBDD, Ligand-Based Drug Design; LBRD, ligand-based rational design; LOX-5,

arachidonate 5-lipoxygenase; LSTM, Long Short-Term Memory; MAM, mitochondrial associated ER membrane; MAOs, monoamine oxidase; MM, molecular modeling; MTDLs, multitarget directed ligands; nM, nanomolar; Nrf2, Nuclear factor erythroid 2–related factor 2; PCA, principal component analysis; PCs, principal components; PD, Parkinson's disease; PDB, protein data bank; PGRMC1, progesterone receptor membrane component 1; PSO, Particle Swarm Optimization; QSAR, Quantitative Structure-Activity Relationship; ROS, reactive oxygen species; RNNs, Recurrent Neural Networks; SBDD, Structure Based Drug Design; SBRD, structure-based rational design; SMILES, Simplified Molecular Input Line Entry Specification; t-Bu, tert-butyl; THC, tetrahydrocannabinol; TMEM97, Transmembrane Protein 97; VAE, Variational autoencoders; VS, virtual screening; ZBG, zinc binding group.

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